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Title: A Survey of Native Microbial Aggregates on Alfalfa, Clover and Mung Bean Sprout Cotyledons for Thickness as Determined by Confocal Scanning Laser Microscopy

Author(s): W.F. Fett, and P.H. Cooke

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Short communication

A survey of native microbial aggregates on alfalfa, clover and mung bean sprout cotyledons for thickness as determined by confocal scanning laser microscopy ☆

William F. Fett*, Peter H. Cooke

Agricultural Research Service, Eastern Regional Research Center, US Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA

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Abstract

In nature, bacteria are often organized in aggregates or biofilms rather than as solitary cells. Biofilms on inert surfaces have been studied in depth using confocal scanning laser microscopy (CSLM) with a variety of fluorescent probes. In contrast, there have been few studies of native microbial aggregates or biofilms on living surfaces, including plants. In this study we used CSLM in combination with the LIVE/DEAD® BacLight™ Viability Kit (Molecular Probes Inc.) to determine the thickness of native microbial aggregates on alfalfa, clover and mung bean sprouts purchased from retail outlets. A survey of aggregate thickness was made by use of a 20× dry lens primarily due to its large free working distance, broad field of view and the uneven topography and shape of cotyledon surfaces. Values for measured thickness (*z*-axis) were corrected based on the point spread function of fluorescent latex spheres (1.98 μm in diameter). Aggregates consisted primarily of live bacteria. Aggregates on mung bean cotyledons were significantly ($P<0.05$) thicker (average = 6.4 ± 2.1 μm, median = 6.0 μm, range of 3.4–10.6 μm) than those on alfalfa (average = 3.3 ± 2.1 μm , median = 2.8 μm, range = 1.7–12.6 μm) or clover (average = 3.0 ± 1.1 μm, median = 2.8 μm , range = 1.7–5.4 μm). Average thickness was not significantly ($P > 0.05$) different for aggregates on clover and alfalfa. Bacteria in fully hydrated aggregates as imaged by CSLM appeared to be less densely packed when compared to sprout surface biofilms imaged previously in our laboratory with conventional scanning electron microscopy techniques, most likely due to the presence of hydrated bacterial exopolymers. Despite a lack of considerable thickness, aggregates and biofilms on plant surfaces may harbor plant and human pathogens making their eradication more problematic and also protect pathogens and native bacteria from adverse environmental conditions.

Keywords: Alfalfa; Aggregates; Biofilm; Clover; Confocal scanning laser microscopy; Mung bean; Sprouts

1. Introduction

In the natural environment as well as industrial and medical settings bacteria are often present in biofilms (or aggregates) rather than as solitary cells (Costerton et al., 1995; Davey and O'Toole, 2000; Watnick and Kolter, 2000). Biofilms are assemblages of microbes attached to a surface and to each other by a matrix composed of bacterial macromolecules including exopolysaccharide and deoxyribonucleic acid (Sutherland, 2001; Whitchurch et al., 2002). Biofilms can range in structure from monolayers to macroscopic communities and in natural environments usually consist of multiple microbial species (O'Toole et al., 2000; Wimpenny et al., 2000). Most studies on biofilm formation have been conducted after microbial growth on inert surfaces such as glass, plastic and stainless steel. Biofilms were initially studied by use of transmission and conventional scanning electron microscopy with the inherent problems of artifacts induced by the required fixation, dehydration, and staining steps. The advent of confocal scanning laser microscopy (CSLM) techniques has allowed for the in situ non-destructive study of living biofilms in their

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*Corresponding author. Tel.: +1-215-233-6418; fax: +1-215-233-6406.

E-mail address: wfett@errc.ars.usda.gov (W.F. Fett).

natural hydrated state (Caldwell et al., 1992; Takeuchi and Frank, 2001). Optical sections are obtained essentially free from out-of-focus light and 3-D images can be constructed from the series of digitized 2-D images. Along with the use of various fluorescent probes the images can provide much information not only on biofilm architecture but also the biochemical and physical state of microbial cells, their organization and the chemistry of their microenvironment. Mature biofilms can be highly heterogeneous and in flowing systems are often composed of mushroom shaped microcolonies of microbes enclosed in bacterial exopolysaccharide and separated by water channels (Costerton et al., 1999; O'Toole et al., 2000; Stoodley et al., 2002).

Microbial adherence and subsequent biofilm formation are also of great interest in relationship to food science. Pathogenic as well as spoilage micro-organisms present in biofilms are much more difficult to remove physically from foods and processing equipment and bacteria in biofilms may be up to 500-fold or greater in resistance to the effects of antimicrobial chemicals when compared to their free-living counterparts (Bower et al., 1996; Costerton et al., 1995; Frank, 2001; Kumar and Anand, 1998).

In contrast to biofilms present on non-living surfaces, the study of biofilms on the surface of plants including fruits and vegetables is in its infancy. If human pathogens can form homogeneous biofilms or become part of native biofilms, they will be more difficult to remove by physical or chemical means and will be better able to survive adverse environmental conditions. Various studies, most employing traditional scanning electron microscopy (SEM), indicated that native biofilms (or aggregates) are present on a variety of plant surfaces (Carmichael et al., 1999; Fett, 2000; Fett and Cooke, 2003; Fuqua and Matthysse, 2001; Itoh et al., 2001; Monier and Lindow, 2003, 2004; Morris et al., 1997; Sharga, 1997; Sutherland, 1996). Early studies indicated that leaf surfaces in tropical regions could harbor highly heterogeneous biofilms consisting of yeast, fungi, algae and bacterial cells (Dickinson, 1986; Ruinen, 1961). Recent studies utilizing epifluorescence microscopy, traditional SEM and CSLM indicated that highly heterogenous biofilms can occur on field grown leafy vegetables in temperate regions (Morris et al., 1997, 1998, 2002; Morris and Monier, 2003). These aggregates consisted of both Gram-negative and Gram-positive bacteria, yeasts and filamentous fungi.

During the 1990s recorded outbreaks of foodborne illness in the US due to the consumption of alfalfa and clover sprouts contaminated with *Salmonella* spp. or *E. coli* O157 increased dramatically (NACMCF, 1999; Taormina et al., 1999). The first foodborne outbreak of salmonellosis in the US due to mung bean sprouts took place in year 2000. Our previous studies using SEM indicated that native biofilms are abundant on the surfaces of a variety of green (chlorophyll-containing) sprouts including alfalfa, clover, broccoli and sunflower as well as mung bean sprouts which are grown in the dark (Fett, 2000; Fett and Cooke, 2003). Biofilms were most easily observed on cotyledon surfaces as compared to hypocotyls and roots and biofilms covering almost an entire cotyledon surface (up to 2 mm in length) were found. Biofilms were composed of rod-shaped bacteria, cocci-shaped bacteria or yeasts or mixtures thereof and were partially or totally covered with presumably microbial exocellular materials. Filamentous fungi were not members of the observed biofilms on sprouts. On the leaf surface of several plant species, aggregated bacteria have been estimated to comprise between 30% and 80% of the total bacterial populations (Morris and Monier, 2003).

Due to the limitations of traditional SEM for examining biofilms as discussed above, the study reported here employed CSLM to observe microbial aggregates on cotyledons of alfalfa, clover and mung bean sprouts without the need for destructive sample preparation. The primary goal of the study was to determine aggregate thickness. The microbial assemblages imaged in this study are referred to as aggregates rather than biofilms as the presence of microbial exopolymeric substances was not evaluated.

2. Materials and methods

2.1. Sprouts

Alfalfa, clover and mung bean sprouts were purchased from retail outlets. Sprouts that appeared fully turgid and were free from visible fungal and bacterial rots were selected. The packaging did not display sell by, expiration or best used by dates. Sprouts were stored at 4°C until examined (maximum storage time of 1 week, no change in visual appearance noted). Sprouts have a shelf-life of approximately 13–15 days (Price, 1988).

2.2. Confocal scanning laser microscopy

Microscopy was done with a Leica model TCS SP Spectral Confocal Microscope (Leica Microsystems, Heidelburg, Germany) fitted with an inverted microscope (Leica DM IRBE). A 20× dry lens objective (Leica Dry HC PL FLOUTAR) [numerical aperture = 0.5, free working distance = 1.15 mm, *z*-resolution = 1.64 μm] was employed. The objective lens was corrected for use with a coverslip. In preliminary experiments, combined confocal reflection and fluorescence images were obtained by staining with acridine orange, illuminating with the HeNe laser (633 nm) with imaging at 633 nm along with illumination with the Ar

laser and simultaneous imaging in the green channel (500–560 nm) to image stained microbes. Staining was accomplished by immersing whole sprouts in 0.1% acridine orange for 10 min and then rinsing off the excess dye with water. The stained cotyledons were excised from the sprouts and placed onto 10–50 µl drops of sterile water on the cover slip surface of an uncoated MatTek culture dish (35 mm) with a 14 mm microwell (MatTek Corporation, Ashland, Massachusetts, USA). Distilled water was added to the bottom of the dish to inhibit dehydration. Images were processed for display using Photoshop software (Adobe Systems Inc., San Jose, California, USA).

In subsequent experiments cotyledons were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (cat. no. L-7012, Molecular Probes Inc., Eugene, Oregon). The staining kit contains the fluorescent nucleic acid stains SYTO 9 and propidium iodide. The SYTO9 stain (emission maximum of 500 nm) penetrates bacterial cells with compromised or intact plasma membranes, while the propidium iodide stain (emission maximum of 635 nm) penetrates bacteria with compromised plasma membranes only and quenches the green fluorescence of SYTO9. Thus, presumably live cells (intact membranes) fluoresce green while cells with compromised membranes (presumably dead) fluoresce red. To prepare the final stain, equal volumes of the two stains were mixed together and then 3 µl of the mixture was added to 1 ml of sterile distilled water in a 1.5 ml capped plastic tube with vortex mixing for 15 s. Two methods for staining with the viability kit were used. In initial experiments, whole attached cotyledons were submerged in the staining mixture for 10–15 min, excised from the whole sprout and then placed onto the cover slip as stated above. In the survey to determine aggregate thickness, a drop (3–10 µl) of the final stain mixture was placed directly onto the cover slip surface and then the adaxial surface of an excised cotyledon was placed onto the droplet of stain and left at room temperature for at least 15 min before imaging. A flat area of the cotyledon was located using transmitted light and phase contrast optics. Specimens were then illuminated with the Ar laser (488 nm) with simultaneous dual-channel imaging (green channel, detection at 495–520 nm; red channel, detection at 610–635 nm). A survey image was obtained at 1 × zoom to locate well-separated aggregates and then individual biofilms were imaged at 8 × zoom. To confirm that dead cells present on cotyledon surfaces were red fluorescent after staining, detached alfalfa cotyledons were submerged in isopropanol for 10 s and then stained with the LIVE/DEAD® *Bac*Light™ stain as described above. Combined reflection and fluorescence images were also obtained using the LIVE/DEAD® *Bac*Light™ stain. At least six pairs of cotyledons per sprout type were surveyed. Aggregate areas were estimated by tracing the outer edges of the aggregates using the cursor and then the integrated areas were automatically calculated by the application software (Leica confocal software v. 2.5 Build 1104). Only well-separated aggregates were imaged and the edges of aggregates were estimated to be where closely aligned bacteria were no longer present. Aggregate thickness was estimated by first defining the upper and lower boundaries by adjusting focus and then generating image galleries (8 optical slices each, variable thickness) taken at 8 × digital zoom. To confirm that imaged biofilms were present on a flat surface, individual optical sections within each gallery stack were examined to make sure that the individual bacteria observed did not shift in position from one image to another. To compensate for the problem of distortion in the *z*-axis with the 20 × lens (cf. point spread function) (Webb, 1999), correction factors were determined by measuring the diameter of Fluoresbrite® YG Plain Microspheres (1.98 µm diameter, Polysciences Inc., Warrington, Pennsylvania, USA) placed on the cotyledon surfaces.

2.3. Statistical analysis

Data for aggregate thickness was analysed for significant differences ($P < 0.05$) by the use of analysis of variance and the Bonferroni least significant difference (LSD) separation procedure (Miller, 1981).

3. Results and discussion

The estimation of thickness for a large number of microbial aggregates sitting on the plant surfaces was challenging. The use of lenses of higher magnification and *z*-resolution was not feasible for a variety of reasons. There was a need for a large free working distance and broad field of view in order to locate a large number of aggregates (greater than 50 per sprout type) on flat areas of the cotyledons suitable for imaging. In addition, rapid photobleaching was encountered when using a 100 × oil immersion lens in preliminary studies. For the purpose of this study only discrete areas of intense colonization were subjected to analysis of thickness. These areas were considered to be aggregates as the presence of microbial exopolymer materials was not evaluated. Based on our earlier studies using traditional SEM techniques (Fett, 2000; Fett and Cooke, 2003) many, if not most, of these aggregates were likely to be biofilms with microbes bound to each other and to the plant surface by microbial exopolymeric materials. Areas where large numbers of bacteria were confined solely to the junctions of the epidermal cells were not imaged although these bacteria may, on occasion, form extensive contiguous networks (Fig. 1C). The smallest aggregate imaged had an estimated area of

32 μm² while the largest had an estimated area of 7283 μm². Due to distortion in the optical path (Fig. 1C, middle and bottom panels), a correction factor for measurements of aggregate depth was necessary. The mean correction factor as determined from measurements in the *z*-axis from stacks of optical sections of 10 beads was 0.22 (measured depth × 0.22 = actual depth).

Bacterial colonization of sprout cotyledon surfaces ranged from very sparse to very dense even comparing two cotyledons from the same sprout. No filamentous fungi were noted on the surface of the cotyledons. The surface of mung bean cotyledons was more uneven than the surfaces of alfalfa and clover cotyledons making aggregates on their surface more difficult to image. The surface of a lightly colonized alfalfa cotyledon is shown in Fig. 1A. The cell walls of the stomatal guard cells immediately adjacent to the stomatal openings stained red with propidium iodide. On lightly colonized cotyledon surfaces, the majority of bacterial cells were present in the surface depressions at the junctions of epidermal cells. A heavily colonized alfalfa cotyledon surface is shown in Fig. 1B. Combined confocal reflectance and fluorescence imaging allowed for localization of sites of bacterial colonization in regards to plant surface topography (Fig. 1C, top panel). Aggregates consisting of more than a single layer of bacterial cells were observed on alfalfa cotyledon surfaces (Fig. 1D). Clover and mung bean cotyledon surfaces can also harbor large numbers of bacteria (Figs. 2A and C) and, as for alfalfa cotyledons, aggegates consisting of more than a single layer were imaged (Figs. 2B and D). The use of CSLM to demonstrate that hydrated biofilms consisting of more than a monolayer of bacterial cells occur on sprout surfaces confirmed the results of our earlier studies that utilized conventional SEM techniques with critical-point dried samples (Fett, 2000; Fett and Cooke, 2003). The fully hydrated aggregates on sprout surfaces as imaged by CSLM (Figs. 1D, 2B and D) appeared to be less compact than biofilms imaged previously using traditional SEM of critically dried samples. This difference was likely due to the presence in the current study of fully hydrated bacterial exopolymer materials such as exopolysaccharides.

In the survey, a total of 73, 57 and 57 aggregates on cotyledon surfaces of alfalfa, clover and mung bean, respectively, were analysed for thickness. The estimated area of the individual aggregates imaged ranged from 32 to 7283 μm². The aggregates on mung bean cotyledons were of significantly ($P < 0.05$) greater average thickness (average of 6.37 ± 2.10 μm, median of 6.006 μm, range of 3.39–10.63 μm) compared to aggregates on alfalfa (average of 3.34 ± 2.08 μm, median of 2.772 μm, range of 1.69–12.63 μm) and clover (average of 3.01 ± 1.08 μm, median of 2.772 μm, range of 1.65–5.08 μm). The average thickness of aggregates on alfalfa and clover were not significantly different ($P > 0.05$). The maximum thickness of the aggregates on sprout cotyledons was not as thick as biofilms present on leaves of field grown leafy vegetables (up to 20 μm) (Morris et al., 1997, 1998).

The great majority of bacterial cells present in the aggregates imaged in the present study were alive based on their green fluorescence. Webb et al. (2003) recently reported that prophage-mediated cell death plays an important role in differentiation inside of microcolonies of *Pseudomonas aeruginosa* making up biofilms in flowing systems. Bacterial cells at the center of 7–12 day old colonies exhibited cell death and lysis. This phenomenon was not noted for microbial aggregates on sprout cotyledon surfaces in our study possibly due to the relatively minimal thickness of aggregates imaged or to the unique nature of *P. aeruginosa*, a bacterium that is not a common epiphyte of plants.

Most aggregates imaged on sprout cotyledon surfaces in this study were similar in appearance to the flat biofilms formed by *P. aeruginosa* when grown in flow chambers with citrate, benzoate and casamino acids as carbon sources rather than glucose, a carbon source that supported biofilms composed of mushroom shaped microcolonies (Klausen et al., 2003). However, a very few aggregates (Figs. 1D, 2B and D) exhibited a more complex architecture. Our previous SEM study of native biofilms on mung bean sprout cotyledons also indicated that biofilms with three-dimensional structure composed of several layers of bacterial cells are occasionally present (Fett and Cooke, 2003). In the current study the average and median depth of imaged aggregates on mung bean sprout cotyledons was higher than those for alfalfa and clover sprouts. One possible explanation for this observation is that the level of plant nutrients available for microbial growth is somewhat greater on the surface of mung bean sprout cotyledons than on alfalfa or clover cotyledons. It is possible that some 3-D architecture of aggregates on sprouts is lost after harvest and subsequent drying of the plant surfaces. We are in the process of evaluating this possibility by examining aggregates on sprouts grown in our laboratory both pre- and post-harvest using CSLM as well as environmental SEM techniques.

From a food quality and safety perspective, native biofilms on sprout surfaces may act as protected sites for bacterial plant and human pathogens making their elimination by physical or chemical means more difficult. The possibility is supported by the finding that introduced bacteria can cluster with native bacteria in microcolonies on plant root surfaces (Normander et al., 1999). In addition, the presence of microbes in aggregates rather than as solitary cells on plant surfaces can protect them from adverse environmental conditions (Monier and Lindow, 2003).

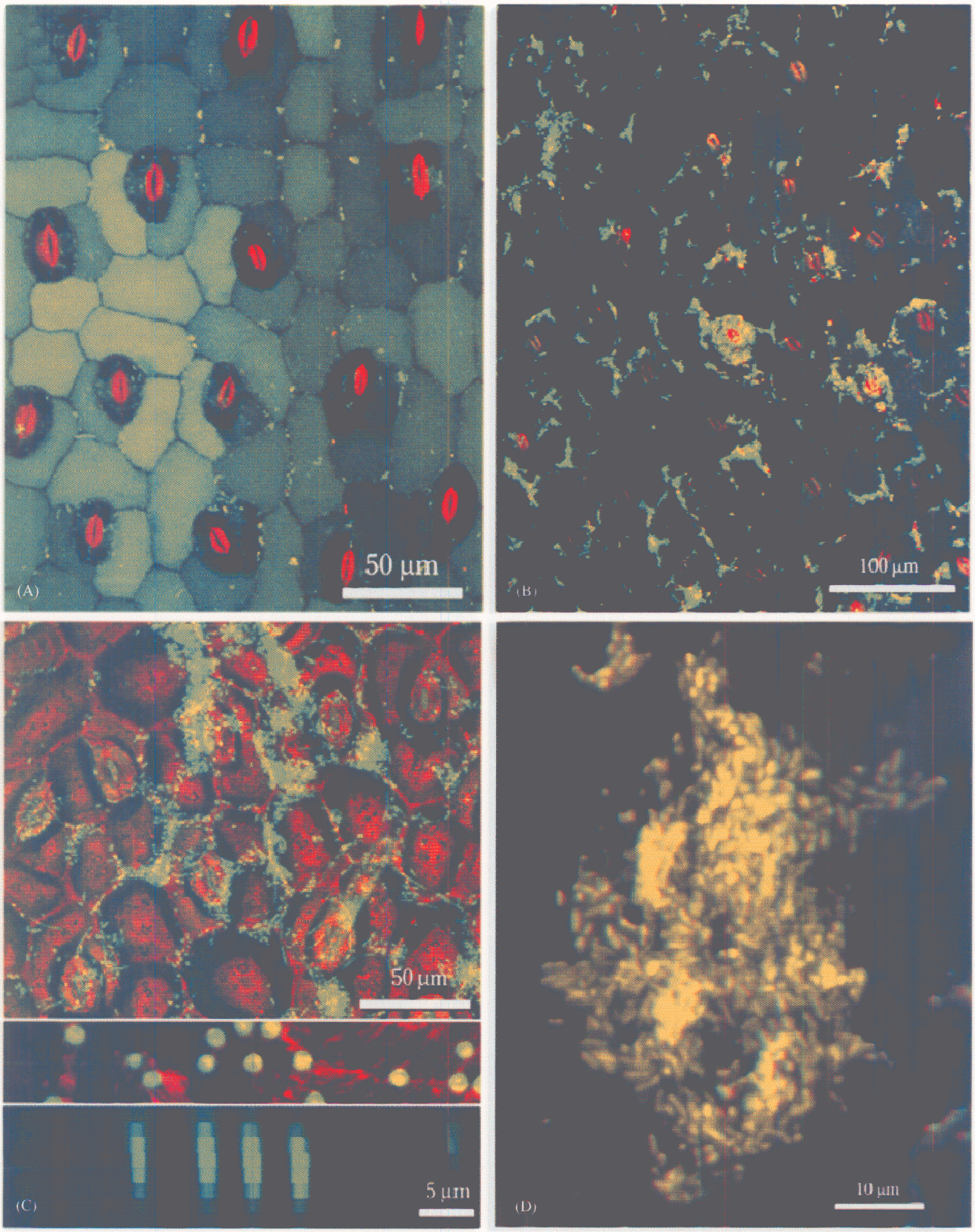

Fig. 1. CSLM images of the adaxial surface of alfalfa sprout cotyledons. (A) A sparsely colonized cotyledon showing the location of stomata (Live/Dead® stain). (B) A more heavily colonized surface with microbial aggregates present (Live/Dead® stain). (C), A combined reflection and fluorescence image showing extensive colonization and surface topography (Acridine Orange stain) (top panel), fluorescent beads in maximum projection (*xy*) (middle panel) and in vertical cross-section (*xz*) (bottom panel) along the line shown in the middle panel. (D) A stereo image of multi-layered aggregate (Live/Dead® stain). The anaglyph stereo image should be viewed with red/green 3-D glasses. For Live/Dead® stained cotyledons, live bacteria (intact membranes) are green fluorescent and dead bacteria (compromised membranes) are red fluorescent. Features in the stereo image are elongated in the *z*-axis as demonstrated in Fig. 1C.

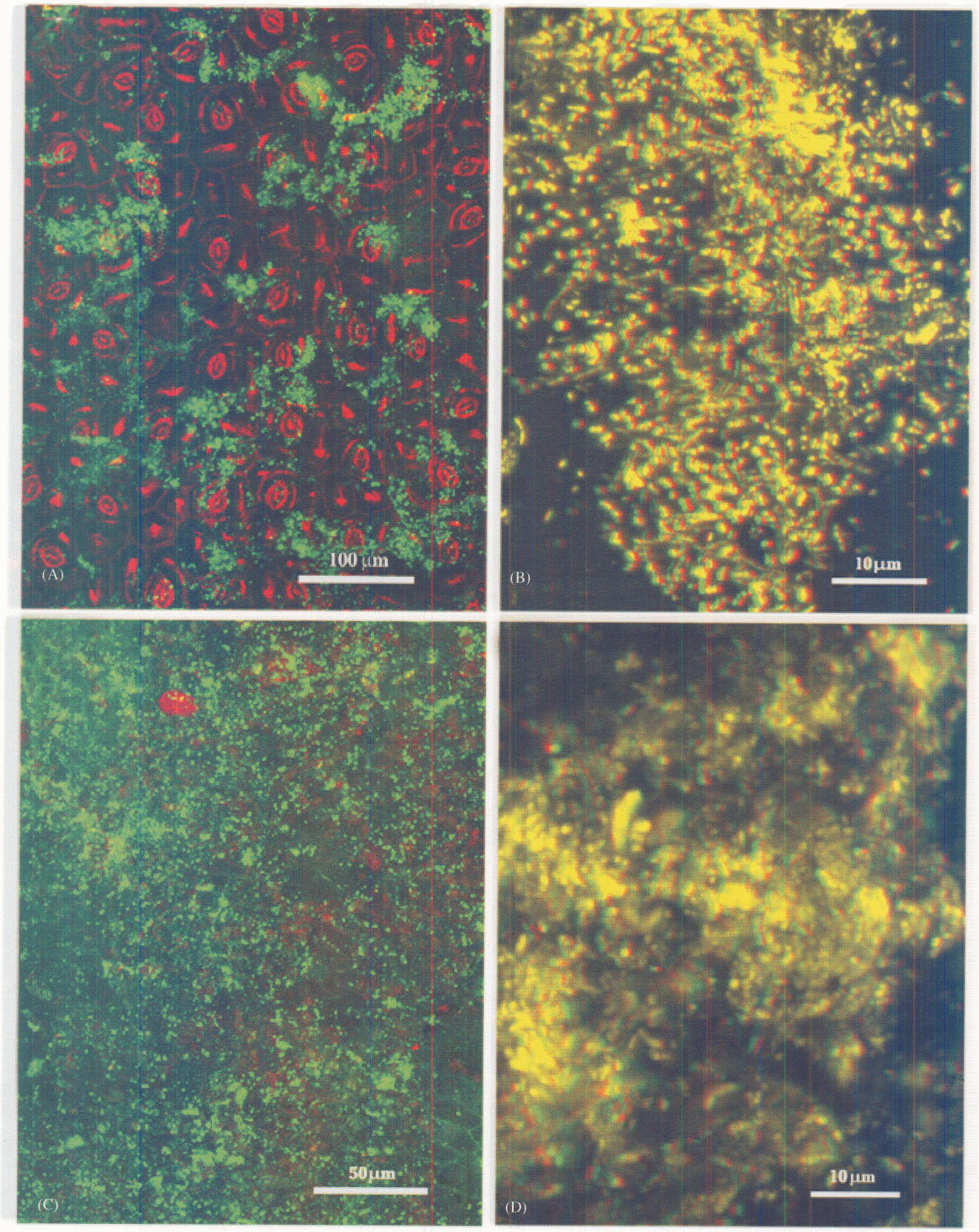

Fig. 2. CSLM images of the adaxial surface of clover (A, B) and mung bean (C, D) cotyledons. (A) A combined reflection and fluorescence image showing extensive colonization and surface topography (Live/Dead® stain). (B) A stereo image of a multi-layered aggregate (Live/Dead® stain). (C) A heavily colonized surface (Live/Dead® stain). (D) A stereo image of a multi-layered aggregate (Live/Dead® stain). The anaglyph stereo images should be viewed with red/green 3-D glasses. For Live/Dead® stained cotyledons, live bacteria (intact membranes) are green fluorescent and dead bacteria (compromised membranes) are red fluorescent. Features in the stereo images are elongated in the z-axis as demonstrated in Fig. 1C.

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